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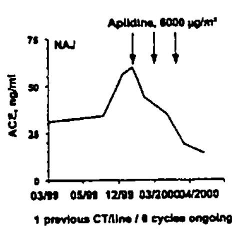
(54) Title: APLIDINE TREATMENT OF CANCERS



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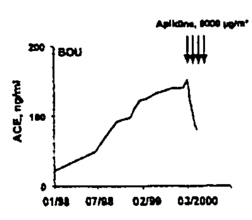

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(57) Abstract: Aplidine demonstrates considerable promise in phase (I) clinical trials for treatment of tumours, and various dosing regimes are given. Tumor reduction has been observed in several tumour types including renal carcinoma, colorectal cancer, lung carcinoid, medullary thyroid carcinomas and melanoma. It has also been found that aplidine has a role in inhibiting angiogenesis, complementing the anti-tumour activity.

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APLIDINE TREATMENT OF CANCERS

The present invention relates to the treatment of cancers.

BACKGROUND OF INVENTION

Cancer comprises a group of malignant neoplasms that can be divided into two categories, carcinoma, comprising a majority of the cases observed in the clinics, and other less frequent cancers, which include leukaemia, lymphoma, central nervous system tumours and sarcoma. Carcinomas have their origin in epithelial tissues while sarcomas develop from connective tissues and those structures that had their origin in mesoderm tissues. Sarcomas can affect, for instance, muscle or bone and occur in the bones, bladder, kidneys, liver, lung, parotid or spleen.

Cancer is invasive and tends to metastasise to new sites. It spreads directly into surrounding tissues and also may be disseminated through the lymphatic and circulatory systems. Many treatments are available for cancer, including surgery and radiation for localised disease, and drugs. However, the efficacy of available treatments on many cancer types is limited, and new, improved forms of treatment showing clinical benefit are needed. This is especially true for those patients presenting with advanced and/or metastatic disease. It is also true for patients relapsing with progressive disease after having been previously treated with established therapies for which further treatment with the same therapy is mostly ineffective due to acquisition of resistance or to

limitations in administration of the therapies due to associated toxicities.

Chemotherapy plays a significant part in cancer treatment, as it is required for treatment of advanced cancers with distant metastasis and often helpful for tumour reduction before surgery, and many anti-cancer drugs have been developed based on various modes of action.

Dehydrodidemnin B, now known as aplidine, is the subject of WO91/04985.

Further information on aplidine is to be found in, for example:

Jimeno, J., "Exploitation of marine microorganisms and invertebrates: Anticancer drugs from marine origin", IBC Conf Discov Drugs from Nat Novel Approaches New Sources (Dec 8-9, London) 1994, 1994

Faircloth, G. et al., "Dehydrodidemnin B (DDM) a new marine derived anticancer agent (MDA) with activity against experimental tumour models", 9th NCI-EORTC Symp New Drugs Cancer Ther (March 12-15, Amsterdam) 1996, Abst 111

Sakai, R. et al., "Structure-activity relationships of the didemnins", Journal of Medicinal Chemistry 1996, 39 (14): 2819

Urdiales, J.L. et al., "Antiproliferative effect of dehydrodidemnin B (DDB), a depsipeptide isolated from Mediterranean tunicates", Cancer Letters 1996, 102(1-2): 31

Faircloth, G. et al., "Preclinical characterization of aplidine (APD), a new marine anticancer depsipeptide (MADEP)", Proc Amer Assoc Cancer Res 1997, 38: Abst 692

Depenbrock, H. et al., "In vitro activity of aplidine, a new marine-derived anti-cancer compound, on freshly explanted clonogenic human tumour cells and haematopoietic precursor cells", British Journal of Cancer 1998, 78(6): 739

Faircloth, G. et al., "Aplidine (aplidine) is a novel marine-derived depsipeptide with in vivo antitumour activity", Proc Amer Assoc Cancer Res 1998, 39: Abst 1551

Faircloth, G. et al., "Preclinical development of aplidine, a novel marine-derived agent with potent antitumour activity", 10th NCI-EORTC Symp New Drugs Cancer Ther (June 16-19, Amsterdam) 1998, Abst 129

Mastbergen, S.C. et al., "Cytotoxicity and neurocytoxicity of aplidine, a new marine anticancer agent evaluated using in vitro assays", 10th NCI-EORTC Symp New Drugs Cancer Ther (June 16-19, Amsterdam) 1998, Abst 131

In preclinical studies, aplidine had dose-dependent cytotoxic activity against the two epithelial-like cell lines, CT-1 and CT-2, and the human colon cancer cell line, HT-29. The most proliferative line, CT-2, was the most sensitive to aplidine. In addition the compound decreased ornithine decarboxylase activity in all three cell lines (Lobo C, Garcia-Pozo SG, *et al.* Effect of dehydrodidemnin B on human colon carcinoma cell lines. Anticancer Research. 17: 333-336, Jan-Feb 1997). In a similar study, aplidine 50 nmol/L inhibited the growth of the breast cancer cell lines, MDA-MB231 and MCF-7 by 17 and 47%, respectively.

A significant increase in spermidine and spermine was observed in the treated cells (Gomez-Fabre PM, De Pedro E,, et al). Polyamine contents of human breast cancer cells treated with the cytotoxic agents chlorpheniramine and dehydrodidemnin B. Cancer Letters. 113: 141-144, 26 Feb 1997). Flow cytometric analysis showed that aplidine did not induce any apparent cell cycle pertubations (Erba E, Balconi G, et al. Cell cycle phases pertubations induced by new natural marine compounds. Annals of Oncology. 7 (Suppl. 1): 82, 1996). In mice, aplidine was active against implanted P388 leukaemia and B16 melanoma, with an optimal dose of 160 micro/kg. Unlike didemnin B, aplidine was active in SC implanted lewis lung carcinomas (Faircloth G, Rinehart K, *et al.* Dehydrodidemnin B a new marine derived anticancer agent with activity against experimental tumour models. Annals of Oncology. 7 (Suppl. 1): 34, 1996).

Continuous exposure to low concentrations of aplidine inhibited the growth of a number of tumour cell lines, including non-Hodgkin's lymphoma, melanoma and breast, melanoma, ovarian and non-small cell lung cancers. The magnitude of effect was dependent on the time of exposure and appeared to be achievable at non-myelotoxic concentrations. Non-small cell lung cancer, breast cancer and melanoma cell lines were sensitive to a continuous exposure to aplidine at concentrations of >= 0.001 micromol/L. Aplidine had similar toxicity to doxorubicin against clonogenic haematopoietic stem cells (Depenbrock H, Peter R, *et al.* In vitro activity of aplidine, a new marine-derived anti-cancer compound, on freshly explanted clonogenic human tumour cells and haematopoietic precursor cells. British Journal of Cancer. 78: 739-744, No. 6, Sep 1998).

Aplidine had significant activity against mice bearing human cancer xenografts. At a maximum tolerated dose of 2.1 mg/kg, aplidine

produced near complete remissions in some animals with a treated/control (T/C) tumour ratio of 9%. At 1.25 mg/kg, significant activity was seen against gastric tumours (T/C 14%) and prostate tumour growth inhibition was also observed (T/C 25%) (Faircloth G, Grant W, *et al.* Preclinical development of aplidine, a novel marine-derived agent with potent antitumour activity. Annals of Oncology. 9 (Suppl. 2): 34, 1998).

SUMMARY OF INVENTION

We have developed methods to treat human patients with aplidine leading to clinical improvement.

EMBODIMENTS OF THE INVENTION

Thus, the present invention provides a method of treating any mammal, notably a human, affected by cancer which comprises administering to the affected individual a therapeutically effective amount of aplidine, or a pharmaceutical composition thereof.

The present invention also relates to pharmaceutical preparations, which contain as active ingredient aplidine, as well as the processes for their preparation.

Examples of pharmaceutical compositions include liquid (solutions, suspensions or emulsions) with suitable composition for intravenous administration, and they may contain the pure compound or in combination with any carrier or other pharmacologically active compounds.

Administration of the compounds or compositions of the present invention is based on a Dosing Protocol preferably by intravenous infusion. We prefer that infusion times of up to 72 hours are used, more preferably 1 to 24 hours, with about 1, about 3 or about 24 hours most preferred. Short infusion times which allow treatment to be carried out without an overnight stay in hospital are especially desirable. However, infusion may be around 24 hours or even longer if required. Infusion may be carried out at suitable intervals with varying patterns, illustratively once a week, twice a week, or more frequently per week, repeated each week optionally with gaps of typically one week.. Further guidance is given later in this text.

The correct dosage of the compound will vary according to the particular formulation, the mode of application, and the particular *situs*, host and tumour being treated. Other factors like age, body weight, sex, diet, time of administration, rate of excretion, condition of the host, drug combinations, reaction sensitivities and severity of the disease shall be taken into account. Administration can be carried out continuously or periodically within the maximum tolerated dose.

The compound aplidine and compositions of this invention may be used with other drugs to provide a combination therapy. The other drugs may form part of the same composition, or be provided as a separate composition for administration at the same time or a different time. The identity of the other drug is not particularly limited, and suitable candidates include:

a) drugs with antimitotic effects, especially those which target cytoskeletal elements, including microtubule modulators such as

taxane drugs (such as taxol, paclitaxel, taxotere, docetaxel), podophylotoxins or vinca alkaloids (vincristine, vinblastine);

b) antimetabolite drugs (such as 5-fluorouracil, cytarabine, gemcitabine, purine analogues such as pentostatin, methotrexate);

c) alkylating agents or nitrogen mustards (such as nitrosoureas, cyclophosphamide or ifosphamide);

d) drugs which target DNA such as the antracycline drugs adriamycin, doxorubicin, pharmorubicin or epirubicin;

e) drugs with target topoisomerases such as etoposide;

f) hormones and hormone agonists or antagonists such as estrogens, antiestrogens (tamoxifen and related compounds) and androgens, flutamide, leuprorelin, goserelin, cyprotrone or octreotide;

g) drugs which target signal transduction in tumour cells including antibody derivatives such as herceptin;

h) alkylating drugs such as platinum drugs (cis-platin, carbonplatin, oxaliplatin, paraplidineatin) or nitrosoureas;

I) drugs potentially affecting metastasis of tumours such as matrix metalloproteinase inhibitors;

j) gene therapy and antisense agents;

k) antibody therapeutics;

l) other bioactive compounds of marine origin, notably kahalalide F or the ecteinascidins such as et-743;

m) skeletal muscle protectors such as carnitine supplements;

o) other drugs which combat side effects of aplidine such as antiemetics;

p) more generally drugs which allow aplidine to be dosed at the Recommended Dose and manage toxicity.

We have further found that aplidine inhibits expression of the gene (FLT1) encoding the receptor of the Vascular Endothelial Growth Factor (VEGF).

In addition, aplidine has been found to severely inhibit production of the VEGF protein itself by tumour cells.

VEGF secretion by a cell mass, in particular a tumour cell mass, causes *de novo* vascularization (angiogenesis) leading to new blood vessels forming towards the cell mass and establishing a network of capillaries that is able to supply it with irrigation for its sustained proliferation. These effects, in particular the demonstrated abolition of production of VEGF by tumour cells are expected to severely inhibit the ability of the tumour cells to bring forth angiogenesis. In addition, VEGF is required directly by some hematopoietic tumour cells (such as MOLT4 human leukaemia cells) as a growth factor.

Thus aplidine can be predicted to have an inhibitory effect on *de novo* vascularization of growing primary tumours or metastases, therefore inhibiting growth of the tumours, which are known to require vascularization for growth. Aplidine should also be active on hematopoietic tumours.

In addition Aplidine is known to modulate Calcium channel function in cells.

Bladder tumours are one type of tumour over-expressing the receptor to Epithelial Growth Factor (EGF), which leads to upregulation of VEGF and the VEGF receptor. Binding of VEGF to its receptor is believed to lead to cell growth stimulation by means of transitory local calcium ion changes among other mechanisms for signalling. A compound inhibiting VEGF action is expected to be inhibitory to such tumours.

Experimentally, aplidine has been found to have exceedingly high activity on human bladder cancer (giving complete remissions in some animal models), in accordance with the prediction.

Aplidine can be predicted to have a broad spectrum antitumour activity due to its effects on a large number of tumours.

The effect of VEGF is more relevant because it involves an inhibition of new blood vessels. In addition to effects on blood vessels, certain tumours required VEGF directly for cell growth (i.e. leukaemia, lymphomas, bladder tumours and ovarian tumours).

According, we provide a method of treatment of an angiogenic condition which involves administration of aplidine. In particular, we provide a method of treating a tumour that is dependent on the angiogenic process. The invention further provides a process for preparing an inhibitor of angiogenesis, cancer invasion or cancer metastasis comprising admixing an effective dose of aplidine with a pharmaceutically acceptable carrier.

We also provide a method of treating non-tumour related angiogenesis-related disorders, such as retinopathy.

In particular, we envisage methods of treating angiogenesis disorders such as neoplasia including metastasis; ophthalmic conditions such as corneal graft rejection, ocular neovascularization, retinal neovascularization, diabetic retinopathy, retrolental fibroplasia and neovascular glaucoma; ulcerative diseases such as gastric ulcer; and other conditions such as infantile hemangiomas, angiofibroma of the nasopharynx and avascular necrosis of bone; and disorders of the femal reproductive system such as endometriosis.

Responses in cancer patients have been observed in clinical trials with aplidine, demonstrating usefulness of the method of treatment.

Phase I clinical studies and pharmacokinetic analysis demonstrate that aplidine presents a positive therapeutic window with manageable toxicity in the range of dosage required for clinical efficacy in the treatment of cancer patients.

The method consists of administration of drug by intravenous infusion over a period of 72 hrs or less at the recommended dose level (RD) with or without combination with other therapeutic agents.

Aplidine is supplied and stored as a sterile lyophilised product, consisting of aplidine and excipient in a formulation adequate for therapeutic use.

Solubilised aplidine shows substantial degradation under heat and light stress testing conditions, and a lyophilised dosage form was developed, see WO99/42125 incorporated herein by reference. In a currently preferred embodiment freeze-drying was performed from a 500 mg/mL solution of aplidine in 40% (v/v) *tert*-butanol in Water for Injection (WfI) containing 25 mg/mL D-mannitol as bulking agent. The prototype, containing 500 mg aplidine and 25 mg D-mannitol as bulking agent per vial was found to be the optimal formulation in terms of solubility, length of lyophilisation cycle and dosage requirements in the clinical studies. The optimal reconstitution solution was found to be 15/15/70% (v/v/v) Cremaphor EL/ethanol/WfI (CEW). Both reconstituted product and dilutions (up to 1:100 v/v) of the reconstituted product with normal saline appeared to be stable for at least 24 hours after preparation. Shelf-life data, available thus far,

show that the formulation is stable for at least 1 year when stored at 4°C in the dark.

Preparation of the infusion solution is also performed under aseptic conditions by withdrawing the reconstituted solution volume corresponding to dosage calculated for each patient, and slowly injecting the required reconstituted solution volume into an infusion bag or bottle containing between 100 and 1000 ml of 0.9% sodium chloride, after which the whole is homogenised by slow manual shaking. The aplidine infusion solution should be administered intravenously, as soon as possible, within 48 hours after preparation. PVC and polyethylene infusion systems, as well as clear glass are preferred container and conduit materials.

The administration is performed in cycles, in the preferred application method, an intravenous infusion of aplidine is given to the patients the first week of each cycle, the patients are allowed to recover for the remainder of the cycle. The preferred duration of each cycle is of either 3 or 4 weeks; multiple cycles can be given as needed. The drug may also be administered each of the first days of each cycle. Dose delays and/or dose reductions and schedule adjustments are performed as needed depending on individual patient tolerance of treatments, in particular dose reductions are recommended for patients with higher than normal serum levels of liver transaminases or alkaline phosphatase, or bilrubin.

The Recommended Dose (RD) is the highest dose which can be safely administered to a patient producing tolerable, manageable and reversibly toxicity according to the Common Toxicity Criteria established by the National Cancer Institute, (USA) with no more than 2 out of 6 patients presenting any dose limiting toxicities (DLT).

Guidelines for cancer therapy frequently call for administration of chemotherapeutic agents at the highest safe dose at which toxicity is manageable in order to achieve maximum efficacy (DeVita, V.T. Jr., Hellman, S. and Rosenberg, S.A., Cancer: Principles and Practice of Oncology, 3rd ed., 1989, Lipincott, Philadelphia).

DLTs for aplidine using this method of treatment were determined in clinical studies. These studies established a recommended dose level for differing kinds of dosing protocols.

Aplidine can be safely administered at a dosage level at or below the Recommended Dose (RD).

Infusion is currently the preferred procedure, with typical regimes including the following:

24 hour infusion weekly for a number of weeks, say three weeks, followed by one week rest;

biweekly 24 hour infusion;

1 hour infusion weekly for 3 weeks every 4 weeks;

daily infusion of say 1 hour x 5 days q 3 weeks; and

infusion of say 3 hours every other week.

In particular, intravenous infusion can be carried out as a 24 hours infusion once a week for 3 weeks q 4 weeks. More data is given in Examples 3, 4, 11 and 12. A Recommended Dose of 3750 $\mu g/m^2$/wk x 3 seems to be appropriate. This protocol has been amended and patients will now be treated using a different schedule which seems feasible: 3 hour infusion every 2 weeks with no rest. See Example 12. In the 24 h biweekly study patients are being treated at 7000 $\mu g/m^2$/2wks. See examples 6, 14 and 18. Patients included in the study 1h/wk x 3 every 4 weeks are being treated at doses up to 3600

μg/m²/wk x 3 wks. See examples 13 and 17. Another protocol including patients with the daily 1 hour infusion x 5days every 3 weeks is treating patients at a dose of 1200 μg/m²/d x 5d. When aplidine is used in combination with other therapeutic agents, the dosages of both agents may need to be adjusted.

Previously the principal biological responses reported to the administration of aplidine had been observed in animal or *in vitro* models, known to be notoriously inaccurate concerning their usefulness to predict responses in human patients, or in human patients in experimental settings where an effective, safe method of treatment was unavailable (either the dosage used was a toxic dose significantly elevated over the recommended dose or the administration schedule was not appropriate).

In clinical trials using the method of this invention, appropriate plasma levels were achieved in patients at RD, and most importantly, objectively measurable responses demonstrated evidence of clinical benefit to patients.

Definitions for patient toxicities are adopted from WHO Criteria and the responses determined following WHO Response Criteria.

Objective responses were obtained in patients with advanced and/or metastatic cancers refractory to previous treatments, which included those described in the Examples.

In particular treatment with this method has shown responses in cancer patients with advanced and/or metastatic disease, which

exhibited progressive disease after having been previously treated with established therapies.

A preferred method of this invention therefore involves identifying cancer patients who have been treated for cancer, particularly patients who have received chemotherapy, and treating them with aplidine.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the decrease in flt-1 expression observed using microarrays confirmed by RT-PCR analysis.

Figure 2 shows the decrease in flt-1 mRNA induced by aplidine in MOLT-4 cells.

Figures 3a and 3b show the VEGF-Flt-1 Autocrine Loop in MOLT-4 Cells and the effect of aplidine.

Figure 4 shows aplidine Blocks VEGF Secretion from MOLT-4 Cells.

Figure 5 shows aplidine induced block of VEGF secretion.

Figure 6 shows a strong decrease in the levels of VEGF mRNA in MOLT-4 cells.

Figure 7 shows aplidine dose not decrease the activity of VEGF promoter transfected in MOLT-4 cells

Figure 8 shows aplidine does not block the binding of HIF-1 and AP-1 transcription factors to their consensus DNA sequences present in the VEGF promoter

Figure 9 shows aplidine does not block the binding of HIF-1 transcription factor to their consensus DNA sequences present in the VEGF promoter.

Figures 10a and 10b shows VEGF added in the culture medium of MOLT-4 cells slightly reduced the activity of low concentrations of aplidine while at high concentrations is without effects.

Figure 11 show aplidine is able to reduce the secretion of VEGF from the human ovarian cancer line IGROV-1.

Figure 12 shows aplidine reduces the mRNA levels of VEGF also in the human ovarian cancer line IGROV-1.

Figure 13 shows aplidine does not affect the promoter activity of VEGF measured using the luciferase/renilla reporter gene system.

Figure 14 shows a dose AUC relationship.

Figure 15a and 15b show activity in Medullary Thyroid Cancer: CEA Levels

EXAMPLES OF THE INVENTION

Example 1

Gene expression profile in human leukenic MOLT4 cells treated with the marine comopund aplidine

The early changes in gene expression induced by aplidine in MOLT-4 cells were evaluated by using cDNA expression arrays (Atlas Human Cancer, Clontech). MOLT-4 cells were treated for 1 hour with concentrations of aplidine which inhibit the growth by 50% and total RNA was isolated at 0, 1, 6 and 24 hours after drug wash out. Filters were hybridised with equal amount of 32P labelled cDNA. Analysis of the results was carried out using ATLAS IMAGE 1.0 software. Changes in gene expression greater than 2 fold were taken as significant changes in RNA expression and subsequently confirmed by PCR. A marked time-dependent reduction in the expression of VEGF-R1 (flt-1) was observed and confirmed at RNA level by PCR and at protein level by Western blotting.

Example 2

Correlation of selective antitumour activites of the marine-derived compound aplidine using different model systems

Different model systems were evaluated to provide the basis for further clinical work. Selective antitumour activities were seen against two histologically different solid tumours: human gastric and prostate carcinomas. Potent *in vitro* activity to primary gastric tumour

specimens or Hs746T gastric tumour cells is evident with IC_{50} values of 146 and 450 pM, respectively. A less potent, but no less selective, IC_{50} activity of 3.4 nM was determined against PC-3 prostate tumour cells. *In vitro* activities were evaluated in nude rodents using sc implanted tumour fragments or hollow fibres (HF) containing tumour cells.

Table 1. Optimal Dose and *In Vivo* Activities of aplidine

Tumour	Line	Regimen	sc Model	Animal	Dose (mg/kg)	Activity (%T/C)
Gastric	MRI-H254	qd9.ip	xenograft	mouse	2.1	19%
					1.05	17%
		q4dx3.ip	xenograft	mouse	1.25	18%
		24 hr.iv inf.	HF	rat	0.7	20%
Prostate	PC-3	qd9.ip	xenograft	mouse	1.25	25%
					0.62	30%
		q4dx3.ip	xenograft	mouse	2.10	34%
					1.05	38%
		24 hr.iv inf.	HF	rat	0.70	31%
		5 day, iv inf	HF	rat	0.70	33%

Optimal activities were observed in xenografted gastric (17-20%) and prostate (25-38%) tumours following *ip* administration. Follow-up studies necessitated using rats for *iv* infusions. With this variation, a 24 hr *iv* infusion schedule produced similar activities against HF gastric (20%) and HF prostate (31%) tumour cells. Cytotoxicity was also found using a 5 day *iv* infusion schedule against HF prostate tumour cells (33%). The extended *in vivo* evaluations not only show that there is a strong relative correlation to the *in vitro* cytotoxic profile, but also a

strong correlation with *in vivo* models used to characterise the tumour selectivity that identified aplidine as a candidate for clinical development.

Example 3

A phase I and pharmacokinetic study of aplidine given as a weekly 24 hours infusion in patients with advanced solid tumours

In vivo studies revealed that *in vivo* activity increased by prolonging infusion duration. In this study 16 patients were treated. patients characteristics: median age 55 years, median PS 1, male/female 11/5, tumour types being as follows: Head and neck 5, kidney 2, colon 3, rectum 2, sarcoma 1 and melanoma 3, all pre-treated with chemotherapy (median 2 lines).

Aplidine was administered as a 24 h infusion at the following dose levels (Dls): 133 (3 pts), 266 (3 pts), 532 (3 pts), 1000 (3 pts), 2000 (3 pts) and 3000 (1 pt) mcg/m^2/wk x 3 every 28 days.

No dose limiting toxicities (DLTs) were observed. Only mild non-haematological toxicities consisting of nausea g 1, mucositis g 1, asthenia g 1 were reported. Phlebitis of the infusion arm was common and concentration-dependent. Pharmacokinetic analysis was performed in all patients, showing plasma levels at the DLs 1000 mcg/m^2/w and 2000 mcg/m^2/w equivalent to the active *in vitro* concentration (1 ng/ml). At DL 532 mcg/m^2/w clinical improvement was observed in 1 pre-treated patient with advanced melanoma.

Example 4

Phase I and pharmacokinetic (PK) study of aplidine (APL) using a 24 hour, weekly schedule.

To date, 25 pts (median age 58 yrs, median ECOG 1) with advanced, previously treated solid tumours or lymphoma have been treated in this phase I study. Using a schedule of APL 24 h weekly x 3 followed by 1 wk rest, the following dose levels have been tested: 133 (n-3 pts), 266 (3), 532 (3), 1000 (3), 2000 (3), 4500 (3) and 3750 µg/m²/wk (3). With 60 cycles (180 infusions) administered, all pts are evaluable for toxicity. The Maximum Tolerated Dose was 4500 µg/m²/wk x 3 with grade (G) 3 muscular toxicity (biopsy proven type II muscular atrophy). G4 CPK and G3 transaminitis the dose-limiting toxicities in 2 or 4 pts. G2 malaise was observed in most pts and G2/3 emesis at ≥ 2000 µg/m² phlebitis is common but concentration-dependent. All pts have been sampled for PK analysis by LC/MS/MS. Preliminary data indicate extensive tissue distribution, a long elimination t ½ of 10-24 h nad plasma levels > 1 ng/ml (which is active *in vitro*). One pt with advanced melanoma resistant to DTIC/interferon had a clinical improvement maintained for > 30 weeks. This weekly infusional study demonstrates the feasibility and activity of a dose-dense APL schedule. As expected, neuromuscular toxicity is dose-limiting. The possible recommended dose of 3750 µg/m²/wk x 3 is being assessed.

Example 5

Clinical pharmacokinetics (PK) of aplidine (APL) in patients with solid tumours and non-Hodgkin lymphomas.

Four intravenous schedules are under phase I evaluation: weekly 24 h infusion, biweekly 24 h infusion and 5 consecutive days 1 h infusion every 3 weeks. APL blood concentrations are analysed by liquid chromatography-tandem mass spectrometry. Initial results show accumulation in blood cells and a plasma PK characterised by na extensive distribution (volume of distribution usually in excess of 200 L/m^2) and elimination half lives in the order of 10 to 24 h. An open 2-compartment model fits appropriately most profiles after 24 h infusion. For 1 h infusions a 3-compartment model provides a better fit in most cases. Obtained plasma levels are known to be active *in vitro*. Evaluation of additional patients, and a comparison of blood cells and plasma PK is ongoing.

Example 6

Preliminary results of a phase I and pharmacokinetic study of aplidine given as a 24 hour infusion every 2 weeks in patients with solid tumours and non-Hodgkins lymphomas.

Aplidine is given as a 24 hour infusion every 2 weeks. The starting dose was 200 μg/m^2/d and dose escalation proceed including so far 400, 800, 1600, and 3200 μg/m^2/d. A total of 18 patients (M/F: 7/11, median age 52, OMS 0/1: 10/8) have been entered. So far, no dose limiting toxicity was observed. Among evaluable patients, toxicity consisted of mild grade I-II nausea/vomiting, grade I-II asthenia, and cramping occurring during or immediately after the infusion. No neuromuscular toxicity were reported at the evaluated doses. One patient with an advanced lung cancer and documented tumour progression at the dose of 1600 μg/m^2 developed a haemolytic anaemia and thrombocytopenia that was considered unlikely related to the study

drug. Initial pharmacokinetic analysis showed that the drug is extensively distributed and blood concentrations. An open 2-comarptment model fits appropriately most plasma concentration profiles. The terminal half-life is usually in the order of 10-24 h. Clinical improvement was seen in a patient with a non-Hodgkin lymphoma. The accrual is ongoing to determine the dose limiting toxicity and the dose to be recommended in phase II studies.

Example 7

Gene expression profile in human lekemic MOLT4 cells treated with the marine compound aplidine.

In the present study we have evaluated the early changes in gene expression induced by aplidine in MOLT-4 cells by using cDNA expression arrays (Atlas Human Cancer, Clontech). MOLT-4 cells were treated for 1 hour with concentrations of aplidien which inhibit the growth by 50% and total RNA was isolated t 0, 1, 6 and 24 hours after drug wash out. Filters were hybridised with equal amounts of 32P labelled cDNA. Analysis of the results was carried out using ATLAS IMAGE 1.0 software. Changes in gene expression greater than 2 fold were taken as significant changes in RNA expression and subsequently confirmed by PCR. A marked time-dependent reduction in the expression of VEGF-R1 (flt-1) was observed and confirmed at the RNA level by PCR and at the protein level by Western blotting. Whether the down-regulation of flt-1 is involved in the cytotoxic and antitumour effect of aplidine is under investigation at the moment. Also the characterisation of the expression of other genes which appear to be down-regulated after aplidine exposure is currently underway.

Quantitative Changes in Gene Expression Induced by Aplidine in MOLT-4 Cells

Gene	1 Hour	6 Hours	24 Hours
B-RAF	-	+2.0	+2.6
FLT-1	-1.5	-5.0	-3.4
FMS	-	+2.7	+2.1
ETR	+2.7	-	-
DNA-PK	-	+3.0	+3.8
PLK-1	-	+3.0	+4.4

The decrease in flt-1 expression observed using microarrays was confirmed by RT-PCR analysis, see Figure 1.

By using Rnase protection we quantitated the decrease in flt-1 mRNA induced by 20 nM aplidine in MOLT-4 cells, seeFigure 2.

Figures 3a and 3b show the VEGF-Flt-1 Autocrine Loop in MOLT-4 Cells and the effect of aplidine in the VEGF-Flt-1 Autocrine Loop.

Aplidine Blocks VEGF Secretion from MOLT-4 Cells, see Figure 4. Cells were treated for 1 h with 20 nM aplidine. VEGF secreted in the medium was measured by ELISA at the end of treatment and after 6 and 24 hours incubation in drug-free medium.

Aplidine induced block of VEGF secretion is concentration-dependent and is observable already at 5nm, see Figure 5.

By using Rnase protection, a strong decrease in the levels of VEGF mRNA in MOLT-4 cells was observable after 20nM aplidine, see Figure 6.

Aplidine dose not decrease the activity of VEGF promoter transfected in MOLT-4 cells, see Figure 7. Cells were transfected with the VEGF promoter (spanning the first 1000 bases upstream the starting site linked to luciferase reporter gene and with a control plasmid containing the renilla reporter gene. Cells were then treated with different concentrations of aplidine and luciferase activity was measured after 24 hours and compared to renilla activity.

Aplidine does not block the binding of HIF-1 and AP-1 transcription factors to their consensus DNA sequences present in the VEGF promoter, see figure 8. Nuclear extracts were incubated with different aplidine concentrations and labelled oligonucleotides for 60 min. Using gel retardation assay the binding of HIF-1 or AP-1 has been monitored.

Aplidine does not block the binding of HIF-1 transcription factor to their consensus DNA sequences present in the VEGF promoter, see figure 9. Nuclear extracts obtained from cells treated with different aplidine concentrations were incubated with labelled oligonucleotides for 60 min. Using gel retardation assay the binding of HIF-1 has been monitored.

VEGF (10 ng/ml) added in the culture medium of MOLT-4 cells cultured in 10% FCS slightly reduced the activity of low concentrations of aplidine while at high concentrations is without effects, see Figures 10a, 10b.

Aplidine is also able to reduce the secretion of VEGF from the human ovarian cancer line IGROV-1, see figure 11.

Aplidine reduces the mRNA levels of VEGF also in the human ovarian cancer line IGROV-1, see figure 12.

Aplidine does not affect the promoter activity of VEGF measured using the luciferase/renilla reporter gene system, see figure 13.

Example 8

Aplidine blocks VEGF secretion and VEGF/VEGF-R1 autocrine loop in a human leukemic cell line

Aplidine was found to induce strong apoptosis in the human leukaemia cell line MOLT-4. In the same cell line microarray analysis revealed changes in the expression of different genes at early times after treatment. Among these we found that the VEGF-R1 (flt-1) was downregulated by drug treatment and its downregulation was confirmed by northern and western blotting analysis. Further studies showed that treatment of the same cellular system with the compound resulted in a strong reduction of VEGF secretion in the medium. The decrease in VEGF secretion was associated to a decrease in the mRNA encoding for VEGF in MOLT-4 cells treated with aplidine. Trying to elucidate the mechanism by which aplidineidien blocks VEGF secretion, we found that the compound does not change the half life of VEGF mRNA. Similarly, using the electromobility shift assay, aplidine did not change the ability of two transcription factors, HIF-1 and AP-1 to bind their respective consensus sequence present in the promoter of VEGF and did not decrease the transcription of VEGF when a VEGF promoter-luciferase construct was used in transient transfection experiments. The decreased secretion of aplidine was associated with an increased intracellular accumulation of VEGF strongly suggesting that the

compound could act through a block of the secretion of VEGF. Simultaneous treatment of MOLT-4 cells with low concentrations of aplidine and VEGF partially abolish the activity of aplidine suggesting that the drug could partially exert its activity in this cellular system by blocking the autocrine loop VEGF/VEGF-RI.

Example 9

In vitro safety profile of aplidine, a marine natural product with chemotherapeutic potential

Using the CellTiter96 (MTS, Promega) *in vitro* cytotoxicity assay, aplidine exhibits little liver (AML-12) or cardiac toxicity (H9 c2 (2-1); LD_{50} of 1 μM). In contrast, aplidine is very toxic to skeletal muscle (L8), and kidney (NRK-52E) cells (LD_{50} of 0.1 nM), with intermediate toxicity to myelogenous stem cells (FDC-P1, LD_{50} of 0.1 μM) in close agreement with clinical toxicity data. In fact, the dose limiting toxicity in humans is skeletal muscle atrophy.

Aplidine exhibits neurotoxicity at higher *in vitro* concentrations. Using a fluorescent viability stain (ethidium homodimer and calcein AM, Molecular Probes) coupled with immunocytochemistry, we observed that approximately 1 μM aplidine is toxic to brain cells (both to neurons and astrocytes) and motor (choline acetyl transferatse positive) neurons in the spinal cord, but not substance P positive sensory neurones. The motor neuron sensitivity may help to explain the type II muscle atrophy observed (as predicted) in a small group of patients where the AUC concentration of the drug is elevated due to diminished excretion.

Example 10

Phase I study of aplidine in a 5 day bolus q 3 weeks in patients with solid tumours and lymphomas

The objectives are to determine the Maximum Tolerated Dose, the Dose Limiting Toxicity (DLT), the pharmacokinetics (PK) and the recommended dose for phase 2 studies that can be given in a daily 1 hour IV infusion x 5 days q 3 weeks. Patients with solid tumours and low and intermediate grade Non-Hodgkin's Lymphomas were eligible. The daily starting dose of aplidine was 80 $\mu g/m^2$. Cohorts of 3 patients (pts) are treated at each level with dose escalation according to toxicity., 20 patients were treated with 6 dose levels ranging from 80 μg - 720 $\mu g/m^2$, 1 patient is presently at the dose of 960 $\mu g/m^2$. At total of 48 cycles were administered. Non-haematological toxicities were grade 1 and 2 with fatigue reported in most pts. Grade 1 hypersensitivity reactions were documented in 7 patients. Other toxicities included nausea, anorexia, diarrhoea, and anxiety. There were no haematological toxicities. PK analysis was performed in treatment course 1. Aplidine concentrations were analysed by LC tandem mass spectrography. Data suggest dose linear PK with high interpatient variability. The total body clearance was 0.38 L/min and median $t_{1/2}$ of 14.2 hours. Potentially therapeutic plasma concentrations (>1 $\mu g/ml$) were achieved. No objective responses were documented. 1 patient with colon cancer was stable for 9 months and 1 patient with renal cell cancer had a mixed response. In conclusions, no DLT has been documented. Accrual is ongoing at 960 $\mu g/m^2$.

Example 11

Phase I clinical and pharmacokinetic study of aplidine a new marine didemnin, administered as a 24 hour infusion weekly

A phase I trial was performed using 24 hour infusion weekly x 3 followed by 1 wk rest. 32 patients (median age 58 yrs, median ECOG 1) with advanced, previously treated solid tumours have been treated. They have received 64 courses (median/pt: 2 (1-6)) across 8 dose levels: 133 (3 pts), 266 (3 pts), 532 (3 pts), 1000 (3 pts), 2000 (3 pts), 3000 (3 pts), 4500 (4 pts) and 3750 mcg/m^2/wk (10 pts). 2 out of 3 evaluable patients had DLT at 4500 mcg/m^2/wk: grade (G) 4 reversible neuromuscular toxicity (biopsy prevent type II muscular atrophy) and G4 CK increase (1 pt), and transient G3 transaminitis (1 pt). Other toxicities included G 1-2 malaise (most patients treated at ≥3000 mcg/m^2/wk), muscle cramps, G 1-2 emesis (responsive to antiemetics) and injection site reaction (very common and concentration-dependent). All patients have been sampled for PK analysis by a LC/MS/MS method. Pharmacokinetics are linear and the profiles fit a 2 compartment open model. The drug has extensive tissue distribution (Vss=611 L), high clearance (0.47 L/min) and an elimination t 1/2 of 18.8 h. Maintained plasma levels > 1 ng/ml (active *in vitro*) were obtained at doses ≥3000 mcg/m^2. One patient with advanced melanoma resistant to DTIC/interferon had definite clinical improvement maintained for > 30 weeks. Four additional patients had minor responses or stable disease for ≥ 4 months . In conclusion, the DLTs of aplidine administered on a weekly infusion schedule were reversible muscle toxicity and transaminitis, which were observed MTD of 4500 mcg/M^2/wk. The recommended dose for future trials, 3750 mcg/m^2/wk x 3, administered through a central vein catheter, is feasible and associated with mild toxicity.

Example 12

Phase I clinical and pharmacokinetic study of aplidine a new marine didemnin, administered as a 24 hour infusion weekly

Patient Characteristics

Number of patients	35	Tumour type	
Gender (male/female)	23/12	Colorectal	12
Median age, years (range)	56.5 (29-74)	Kidney	6
ECOG performance status		Head and neck	5
0	12	Melanoma	4
1	18	Gastric	2
2	5	Breast	1
Previous radiotherapy		Lung	1
Previous chemotherapy		Soft tissue sarcoma	1
None	4	Lymphoma	1
1 regimen	9	Thyroid	1
2 regimens	12	C. unknown origin	1
3 ≥ regimens	10		
Sites of disease			
1	14		
2	12		
≥3	9		

Patient Accrual

Dose level	Dose (mcg/m²/wk)	No. patients	No. Cycles (range)
I	133	3	9 (1-6)
II	266	3	9 (2.5)
III	532	3	10 (2-6)
IV	1000	3	7 (1-4)
V	2000	3	6 (2-2)
VI	3000	3	4 (1-2)
VII	4500	4	5 (1-2)
VIII	3750	13	21+ (1-4)
Total		35	71+

Worst Toxicities Per Patient

Dose level		I	II	III	IV	V	VI	VII (MTD)	VIII (RD)
No. patients		3	3	3	3	3	3	4	13
Nausea	G2					1			2
	G3						1		
Asthenia	G2			1			1	1	3
	G3						1		
Inj. Site reaction			1		1	1	1	1	3
	G2								
Myositis	G3							1	
CPK elevation	G4							1	
Transaminitis	G3							1	2
	G4								1
Hypersensitivity	G3								1

Antitumour Activity

Patient #008 - (Madrid) Highly pre-treated non measurable metastatic melanoma clinical improvement and tumour shrinkage was observed. A biopsy of one of the metastatic lesions revealed no evidence of residual tumour tissue.

Patient #032 - (Madrid) Renal carcinoma: 20% tumour shrinkage

Patient #034 - (Madrid) Thyroid medullary carcinoma. Clinical improvement and SD at lung lymphangitis. Decrease in CEA marker.

Pharmacokinetics

Plasma aplidine concentrations were determined by liquid chromatography/tandem mass spectography with a limit of quantitation of 0.25 ng/mL and a broad linear range up to 16.00 ng/mL

A total of 15 samples were drawn up to 24 hours after the end of infusion

Dose-linear pharmacokinetics

High Cl median ((quartiles) 0.47(0.40-0.56) L/min) and interpatient variability (coefficient of variation of clearance (Cl), 45%)

Intermediate to long half-life (t ½) (median (quartiles) 18.8 (15.3-25.4) h)

Extensive distribution with supraphysiological volume of distribution (Vss) (median (quartiles) 611 (434-733) L)

Blood cell accumulation (2-8 fold as compared to plasma)

Profiles fit a 2-compartment open model

Figure 14 shows the dose AUC relationship.

Conclusions

DLTs of aplidine administered using this schedule were reversible muscle toxicity and transaminitis observed at the MTD of 4500 mcg/m^2/week x 3

The Recommended Dose for future trials, 3750 mcg/m^2/week x 3 is feasible and associated with mild toxicity (mainly mild asthenia)

Phlebitis at the infusion arm was common, concentration-dependent and avoidable by administration through a central vein catheter

No haematological toxicity was observed

PK is characterised by dose-linearity, relatively prolonged body residence of the compound and extensive distribution. Potentially active plasma levels are reached from 2000 mcg/m^2

An additional phase I study investigating an *iv* 3h infusion given every other week is ongoing. Starting dose 3000 mcg/m^2 q 2 weeks.

<u>Example 13</u>

Phase I trial of aplidine given as a 1 hour intravenous weekly infusion in patients with advanced solid tumours and lymphoma

Adult patients with advanced disease, PS<3, and appropriate organ function are considered eligible; patients receive aplidine wk x 3/q4wks. 24 solid tumour patients have been entered: median (m) age 55 y, m ECOG=1, 15/24 patients treated with =>2 treatment cycles. Seven dose levels (DL) from 133 mcg/m^2/wk to 2700 mcg/m^2/wk have been assessed: 102 infusions are evaluable for toxicity (tox). No haematological tox has been reported, vomiting requiring prophylaxis was observed from 800 mcg/m^2/wk. At 2700 mcg/m^2/wk (4 pts), 1 had G3 hiperbilirrubinemia that has been considered to be dose limiting and therefore 2700 mcg/m^2/wk DL is being expanded. All patients are

being sampled for PK analysis (LC-ESI-MS/MS); kinetics are linear, the m Vss=308 L/m^2 CL is high m=0.60 L/mon and the m elimination half life=14.2 h. Plasma levels >1 ng/ml 24 hours after infusion are reachable form 1800 mcg/m^2/wk. Early hints of activity in gastric cancer have been noted (1 patient at 1200 mcg/m^2). A patient with advanced renal cancer resistant to VBL-IFN has had an ongoing objective response (PR lung mets and SD in peritoneal disease) at 2700 mcg/m^2/wk DL. Aplidine appears to be clinically feasible at pharmacologically appropriate dose levels.

Example 14

A phase I and pharmacokinetic study of aplidine given as a 24-hour continuous infusion every other week (q2w) in patietns with solid tumour and lymphoma

Aplidine was given to patients with solid tumour/NHL as a 24 hr infusion/q2w to 35 patients (median: age=51/ECOG=1) with solid tumour (32 pts) or NHL (3 pts). 23/35 patients were previously exposed to ≥3 previous lines of chemotherapy. Nine dose levels (200-7000 μg/m^2/w/q2w) and 65 cycles (120 infusions) were given. No haematiologic toxicity was reported. Toxicity consisted of G2-3 asthenia and emesis in 9-2 patients and 12-1 pts, respectively. G3 nausea/vomiting (≥5000 μg/m^2) was efficiently treated then prevented with 4HT3-regimens. No cardiac toxicity was reported. At the dose of 5000 μg/m^2/w/q2w, 2 patients experienced transient muscle cramps with reversible G3 CPK-MM elevations. Among 9 patients treated at 6000 μg/m^2/w, 3 patients experienced an increase of CPK-MM and aldolase after the 3rd injection of aplidine. CPK elevations were G1-2 and not symptomatic in 2 patients but a G3 CPK elevation with G3

muscular pain and loss of muscle strength (DLT) was reported in 1 pt. This was reversible with no sequel. Muscle biopsy revealed no significant necrosis of mycocytes. Ultrastructural electron microscopy indicated no morphological mitochondria alteration but a lost of the thick myosin filaments. At 7000 μg/m²/q2w, 4 patients have been entered. Pharmacokinetic analysis (LC-ESI/MS/MS) showed that the AUC-increase is linear, with a large Vss=5391/m², a high clearance (332 ml/mm.m²), and a long terminal half life (15-35 h). Plasma concentrations 24 h after the end of infusion at doses ≥3000 μg/m²/q2w are comparable to efficient *in vitro* concentrations (<1 ng/ml). Activity was observed in NHL (1/3 pts), molecular thyroid (2/2 pts), renal (1/5 pts), and neuroendocrine cancer (1 pt). Mechanistic hypothesis and preventive strategies against muscle toxicity are under evaluation.

Example 15

Microarray assay

The human leukaemia cell line MOLT-4 was used for these experiments MOLT-4 cells were treated for 1 hour with 20 nM of aplidine. Total RNA was extracted at the end of treatment and 6 and 24 hours after recovery in drug-free medium.

5 μg of total RNA were retrotanscribed to cDNA in the presence of ^{32}P-dATP. Equal amounts of radioactive probes were hybridised to Atlas Human Cancer Microarrays (Clontech). After washing, filter were exposed and the results analysed using the Atlas Image software. Only a gene expression difference greater than 2 fold between treated and untreated cells were taken into account. RT-PCR and northern

analysis have been performed to confirm the changes in gene expression found with the microarrays.

Results

Aplidine treatment did cause significant changes in gene expression as early as 1 hour after treatment. After 6 and 24 hours of recovery in drug-free medium, the expression of an increased number of genes has been observed.

At the end of treatment the most significant changes were observed in the expression of the early response gene ETR, and in the VEGF-RI/flt-1 gene which were respectively increased and decreased by treatment

ETR levels returned to normal level after 6 and 24 hours, while the levels of flt-1 further decreased by 6 and 24 hours.

Aplidine induced also an increase in the levels of B-RAF and Fms clearly observable 6 hours after recovery in drug-free medium.

The changes observed in these genes were confirmed by either RT-PCR or northern blot analysis.

From microarray analysis, differences in gene expression (not yet confirmed by RT-PCR) were observed for other genes such as PLK-1.

Example 16

Phase I study of aplidine in a 1 hour daily infusion x 5 q 3 weeks in patients with solid tumours and low and intermediate grade non-Hodgkin's lymphomas.

Results

Patients entered	27	
Evaluable for toxicity	24	
Off Study:	18:	
Progressive Disease		14
Symptomatic Progression		2
Death		1
Other (Dr. Decision)		1

Patient Demography (N=23)

Median age		54 (18-73)
Gender	Female: Male	10:13
PS	0:1:2	5:13:5
Malignancy	Colorectal	7
	Breast	2
	Head and Neck	2
	Renal	3
	Other	9
# Prior regimens	0:1:2:3:4+	5:0:6:6:6
Prior therapy	Adjuvant CT	4
	Metastatic CT	19
	RT	12

Patient Entry/Dose Levels

DL	Dose (ug/m^2)	N	# cycles	Evaluable for toxicity
1	80	3	5 (1-2)	3
2	120	3	15 (2-11)	3
3	240	3	9 (2-4)	3
4	360	4	6 (1-2)	4
5	540	3	7 (2-3)	3
6	720	4	4 (1)	4
7	960	4	4 (1)	4
8	1200	2	2 (1)	2

Non Haematological Toxicity

DL	Fever				Fatigue				Diarrhoea				Nausea			
Grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	1	1			1	2			1				2			
2					3				2				1			
3					1	1	1			2			2	1		
4	1					3			1				1		1	
5		1				2	1		2				2		1	
6	1				1			1	1				3			
7						2							1	1		

Non Hematologic Toxicity cont'd

DL	Hyper-sensitivity				Motor neuropathy				Sensory neuropathy				Myalgia/ weakness			
Grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	1	1			3	2			2	1				1		
2	1								2							
3									1				1			
4	1	1			1				1	1						
5	1				1	1			2	1			1			
6			1				1		1							1
7									1							

Haematological Toxicity

DL	Haemoglobin				Granulocytes				Lymphocytes				Platelets			
Grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	1	2							3							
2	2								1	2			1			
3	1	1								2	1		2			
4	2	2							1	2	1					
5	1	1								2	1					
6		2							2	2						
7		2								1	1					

Biochemical Toxicity

DL	Creatinine				SGOT				Bilirubin				CPK			
Grade	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1					1				1							
2					1											
3	2				2											1
4	1				2											
5					2								1			
6					2				1				1			
7																

Pharmacokinetics

PK characterised by dose linearity

High interpatient variability

High plasma clearance (median 0.38 L/min)

Intermediate to long t ½, median 14.2 hours

Therapeutic plasma concentrations (>1 ug/ul) have been achieved

Conclusions

No DLT documented to date

Mild drug related toxicities include fatigue and nausea (easily controlled with anti-emetics)

Occasional HSR independent of pre-treatment

No clear evidence of neuropathy or myopathy

No objective responses documented although some evidence of possible antitumour activity:

- Two patients with heavily pre-treated CRC, one had stable disease for 6+ months (total 13 cycles), and the other had a 40% reduction in the size of the tumor.

- One patient with renal cell cancer and liver mets with mixed responses (10% reduction in sum of tumour measurements)

Escalation continues: present accrual at 1200 ug/m^2

Example 17

Phase I trial of aplidine, given as a 1h IV weekly infusion in patients with advanced solid tumours and non-Hodgkin lymphoma.

Drug Administration

Aplidine was administered as a 1 hour weekly x3 every four weeks

Patient Characteristics

Number of patients	30	Tumour type	
Median age, years (ranges)	53.5 (36-75)	Colorectal	8
ECOG performed status		Lung	5
0	2	Gastric	4
1	21	Renal	3
2	5	Head and Neck	3
Prior radiotherapy	10	Melanoma	2
Prior chemotherapy (No. regimens)		Ovary	2
1	10	Biliary tract	1
2	10	Oesophagus	1
≥3	8	Pancreas	1

Patient Accrual and Dose Escalation

Dose level	Dose (mcg/m²/wk)	No. patients	No. cycles (range)
I	133	3	5 (1-2)
II	266	3	5 (1-2)
III	532	3	5 (1-2)
IV	800	3	6 (2-2)
V	1200	4	7 (1-2)
VI	1800	4	7 (1-2)
VII	2700	8	12 (1-2+)
VIII	3600	2	3 (1-2+)
Total			

*one cycle definition: 3 consecutive weekly infusions with one week rest.

Worst Toxicities Per Patient

Dose level (weekly x3)	133	266	532	800	1200	1800	2700	3600
No. patients	3	3	3	3	4	4	8	2
Nausea/vomiting G2	-	-	-	3	-	3	2	-
Injection site reaction G2	-	1	-	1	1	1	3	-
Asthenia G2(G3)	-	-	1	1(1)	3	2	1(1)	-
Myalgia G2(G3)	-	-	-	-	(1)	-	-	1
CPK elevation G1	-	-	-	1	-	-	-	1
Transaminitis G2(G3)	1	-	(1)	1	-	1	1(4)[1]	-
Bilirrubin G3	-	-	-	-	-	-	1(1)[2]	-
Alk. Phos. G2(G3)	-	(1)	-	-	-	1	(1)[2]	-
Hypertension G1(G3)	(1)	-	-	1	-	-	-	-

[1]one patient with Hepatitis C; 2 cases reversible by day 8 and 1 DLT

[2]DLT

Characterisation of the Dose Limiting Toxicity Reported

Pat #28 - Edinburgh with Oesophageal adenocarcinoma (no liver mets) treated at 2700 μg/m² weekly had delayed recovery of liver enzymes increase (G3 AST; G3 Bilirubin; G3 ALP) precluding the weekly administration of further doses.

Hints of Activity

Pat #16 - (Edinburgh) with Gastric adenocarcinoma primary resistant to FAM. Slight improvement in the lymph node masses around the lesser curve of the stomach, coeliac axis and pelvis with aplidine at 1200 μg/m² weekly (3600 μg/m²).

Pat #23 - (Barcelona) with Kidney carcinoma and pulmonary nodules as the main disease site, primary resistant to VBL + αIFN and to liposomal Doxorubicin. Partial remission in lung nodules and clinical improvement with resolution of dyspnea after 2 infusions of aplidine at 2700 μg/m² weekly (8100 μg/m²).

Pat #29 - (Barcelona) with kidney carcinoma. Clinical improvement after 3 infusion on the evaluation of a supraclavicular adenopathy. Pending evaluation at 2nd cycle.

Pharmacokinetic Data

Dose-linear PK up to the current dose level of 2700 μg/m²

Important interpatient variability: coefficient of variation of CL, 33%

Relatively high plasma CL: median (quartiles) 329 (288-407 mL/min/m²)

Intermediate t ½: median (quartiles) 15.8 (13.3-19.5) h.

Extensive distribution: median (quartiles) Vss, 345 (220-398) L/m²

Preliminary compartmental analysis: profiles are best fit by a first-order 3-compartment model with a very rapid initial phase (median half-life 0,04 h), followed by an intermediate phase (median half-life 1.4 h) and a longer terminal phase (median half-life 20.7 h)

Conclusions

This ongoing study indicates that aplidine given as a 1 hour infusion weekly with one week rest is clinically feasible up to a dose of 3600 mcg/m^2 every week. Bone marrow toxicity has not been reported. Emesis manageable by prophylactic antiemetics. Muscular toxicity consisting of muscular weakness and increased CK have been seen in one patient treated at 3600. Liver toxicity at the highest dose level investigated has been reported in several patients although was DLT in one patient. Pharmacokinetic information indicates that potentially therapeutic levels are achievable in plasma in patients treated from 1200 μg/m^2 onwards.

The feasibility of 3600 mcg/m^2 every week (dose level VIII) is being investigated

Example 18

A phase I and pharmacokinetic study of aplidine, given as a 24h continuous infusion every other week in patients with solid tumours and non-Hodgkin lymphoma

Patient Characteristics

Number of patients	43	Tumour type	
Median age, years (ranges)	52 (18-71)	Lung	6
ECOG performed status		Colorectal	8
0	19	Kidney	5
1	21	Breast	4
2	2	Pancreas	4
Prior radiotherapy	27	Lymphoma	3
Prior chemotherapy (No. regimens)		Ovary	2
1	7	Thyroid	3
2	5	Bone	1
≥3	29	Melanoma	1
		Prostate	1
		Uterus	1
		Mesothelioma	1
		Gastric	1
		Other	2

Patient Accrual and Dose Escalation

Dose level	Dose (mcg/m²/2wks)	No. patients	No. Cycles (range)
I	200	3	5 (1-3)
II	400	3	6 (2-2)
III	800	3	9 (2-4)
IV	1600	6	11 (1-2)
V	3200	3	5 (1-2)
VI	4000	3	8 (2-4)
VII	5000	3	6 (2-2)
VIII	6000	12	26 (1-6+)
IX	7000	7	12 (1-4+)
Total			

*cycle definition: 2bi-weekly infusions

Worst Toxicities Per Patient

Dose level	200	400	800	1600	3200	4000	5000	6000	7000
No. patients	3	3	3	6	3	3	3	12	7
Nausea/ vomiting G2 (G3)	2	1	-	4	(1)	2	1	2	1
Flushing G1	-	-	1	-	1	1	-	-	1
Asthenia G2 (G3)	-	2	-	2(1)	1	1	(1)	6	2
Muscle cramps G1+G2	-	-	2	1	2	2	2	1	-
Muscle pain G1 (G2)	-	-	-	-	(1)	2	1	1(2)	1
Muscle weakness G1 (G2)	-	-	-	-	-	-	1	1(1)	-
CPK elevation (G2 (G3) [G4]	-	-	-	-	-	-	(1) [1]	1(1)	1
Transaminitis	1	-	-	(1)	-	-	1	-	1

Transaminitis G2 (G3)	1	-	-	(1)	-	-	1	-	1
Hypertension G2	-	1	-	-	-	-	-	-	-
Neutrophenia G4	-	-	-	-	-	-	-	-	1
Pain central catheter G2	-	-	-	-	-	2	-	-	-

Characterisation of Muscular Toxicity (DLT)

Pat #27 - Male patient with medullary thyroid carcinoma treated at 6000 μg/m² weekly had symptomatic G3 CPK with G2 muscular pain. Toxicity recovered within 3 weeks after treatment discontinuation.

3 patients *(5000 and 6000 μg/m²)* experienced a minor elevations of CPKs (≥G2), consisting of CPK MM (muscle) increase with no significant elevation of CPK MB (heart). A parallel elevation of the aldolase level was observed. Signs of improvement by using Carnitine supplements as skeletal muscle protectors are being reported. Muscle biopsies were performed in 2 patients; E/M: partial disappearance of thick filaments of myosin.

Pharmacokinetic Data

Aplidine appears to have a dose-linear PK profile (within the constraints imposed by the low sample size)

Relatively high plasma CL: median (quartiles) value 252 (192-415 mL/min/m²)

High interpatient CL variability (coefficient of variation of CL 62%)

Intermediate to long t ½ with a median (quartiles) value of 23.8 (15.7-35.0 h)

Wide distribution, median (quartiles) Vss of 413 (274-638 L/m²)

Preliminary compartmental analysis: plasma profiles are best fit by a first-order 2-compartment model with a rapid initial (median half-life 0.64 h) and a longer terminal phase (median half-life 25.8 h)

Aplidine Mytotoxicity Relationship with Pharmacokinetics

Muscular toxicity has appeared only at high doses and exposures after 24 h infusion

Cmax values after 1 h infusion are already higher than those after 24 h infusion. Hence, a Cmax relationship may be ruled out

The AUC values in the patients with myotoxicity are high but not the maximum

It affected patients with high, sustained plasma concentrations of aplidine. The 3 patients with clear muscular toxicity had t ½ in excess of 44 h as compared to a median t ½ of 25.8 h after 24 h infusion

Figure 15a and 15b show the Activity in Medullary Thyroid Cancer: CEA Levels

Conclusions

Drug induced muscular changes (expected to be the dose limiting toxicity), reported from dose level number III onwards (1800 mcg/m^2 to 5000 mcg/m^2) is dose limiting toxicity at 6000 mcg/m^2 (1/9 pts)

Antitumour activity has been also noted in patients with NHL and renal carcinoma

The study is now investigating the feasibility of 6000-7000 mcg/m^2 every other week by using carnitine supplements as skeletal muscle protectors.

Claims

1. An effective method of treating cancer in a patient which comprises administering aplidine using at no more than a Recommended Dose consistent with Dose Limiting Toxicity data.

2. The method of claim 1, wherein the dosing is in accordance with one of the following protocols:

24 hour infusion weekly for three weeks, followed by one week rest;

biweekly 24 hour infusion;

1 hour infusion weekly for three weeks every 4 weeks;

daily infusion of say 1 hour x 5 days q 3 weeks; and

3 hour infusion every other week.

3. The method of claim 1 where aplidine blocks VEGF secretion and the VEGF/VEGF autocrine loop.

4. The method of claim 1, wherein the aplidine is adminstered as part of a combination therapy.

5. The method of claim 1, wherein the aplidine is administerd in conjunction with a skeletal muscle protector.

6. The method of claim 1, where the patient has already received the standard treatment for his/her cancer disease and the tumour is refractory.

The decrease in flt-1 expression observed using microarrays has been confirmed by RT-PCR analysis

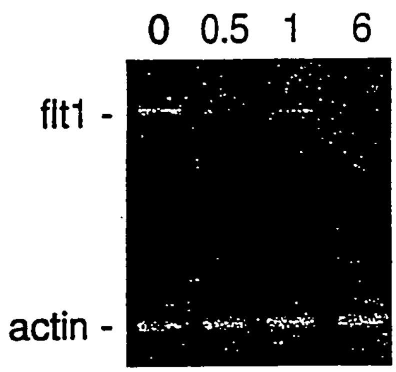


Fig.1

By using RNase protection we quantitasted the decrease In flt-1 mRNA induced by 20nM aplidine in MOLT-4 cells

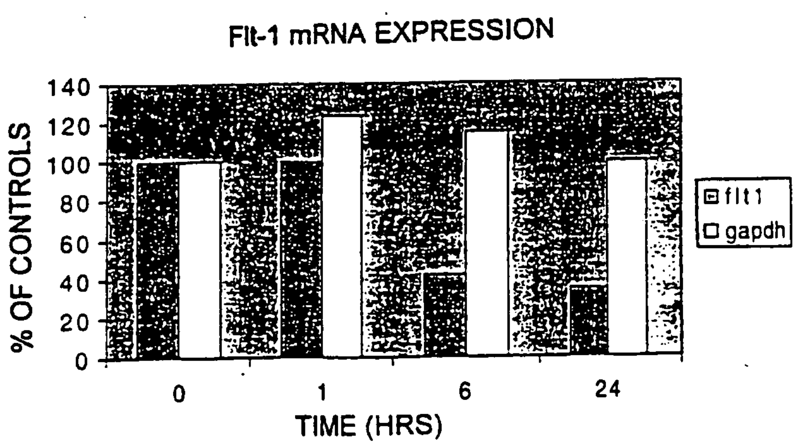


Fig.2

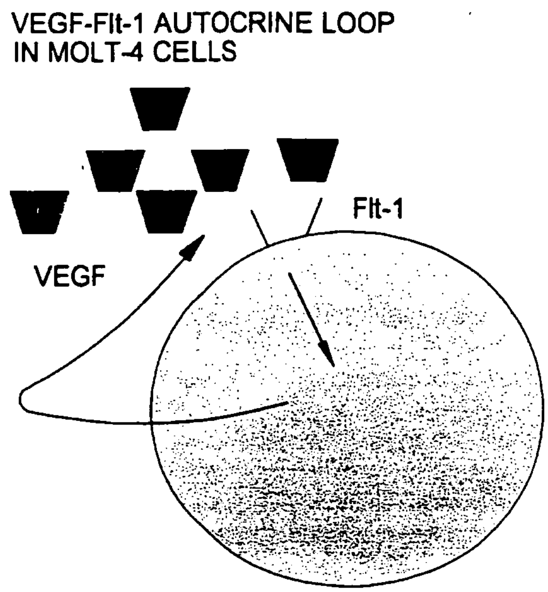

VEGF-Flt-1 AUTOCRINE LOOP
IN MOLT-4 CELLS
Flt-1
VEGF

Fig.3a

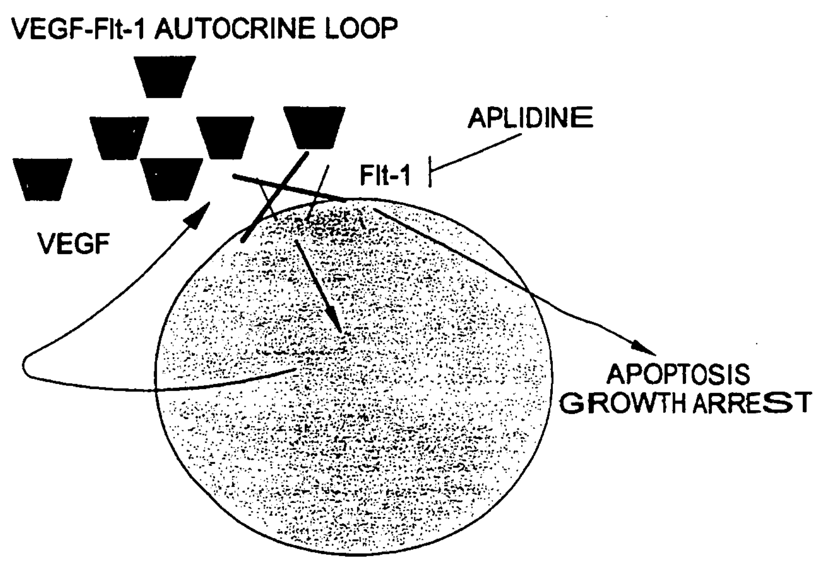

VEGF-Flt-1 AUTOCRINE LOOP
APLIDINE
Flt-1
VEGF
APOPTOSIS
GROWTH ARREST

Fig.3b

APLIDINE BLOCKS VEGF SECRETION FROM MOLT-4 CELLS

CELLS WERE TREATED FOR 1 H WITH 20 NM APLIDINE. VEGF SECRETED IN THE MEDIUM WAS MEASURED BY ELISA AT THE END OF TREATMENT AND AFTER 6 AND 24 HOURS INCUBATION IN DRUG FREE MEDIUM

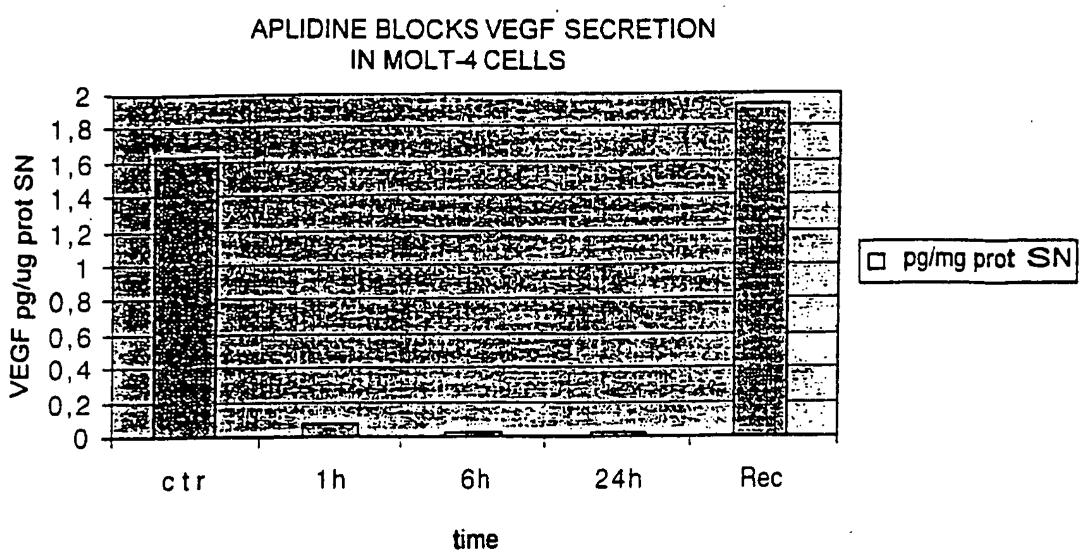


Fig.4

APLIDINE INDUCED BLOCK OF VEGF SECRETIONIS CONCENTRATION-DEPENDENT AND IS OBSERVABLE ALREADY AT 5Nm

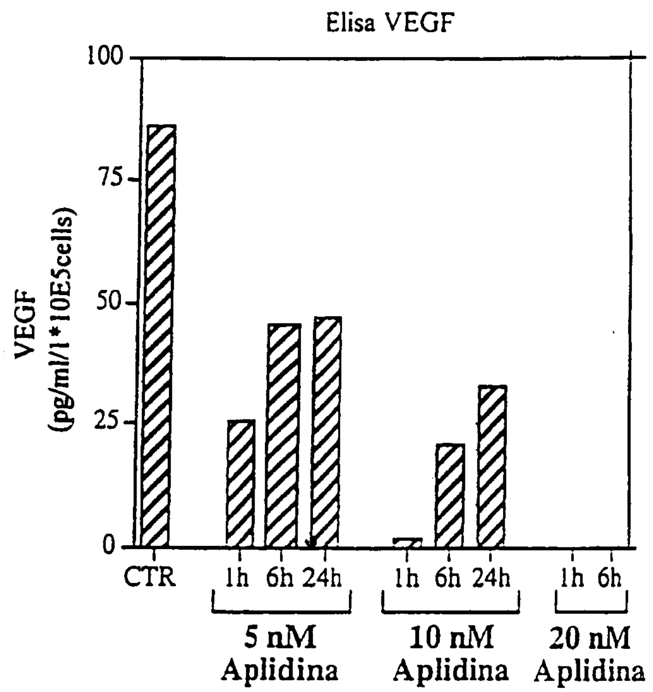
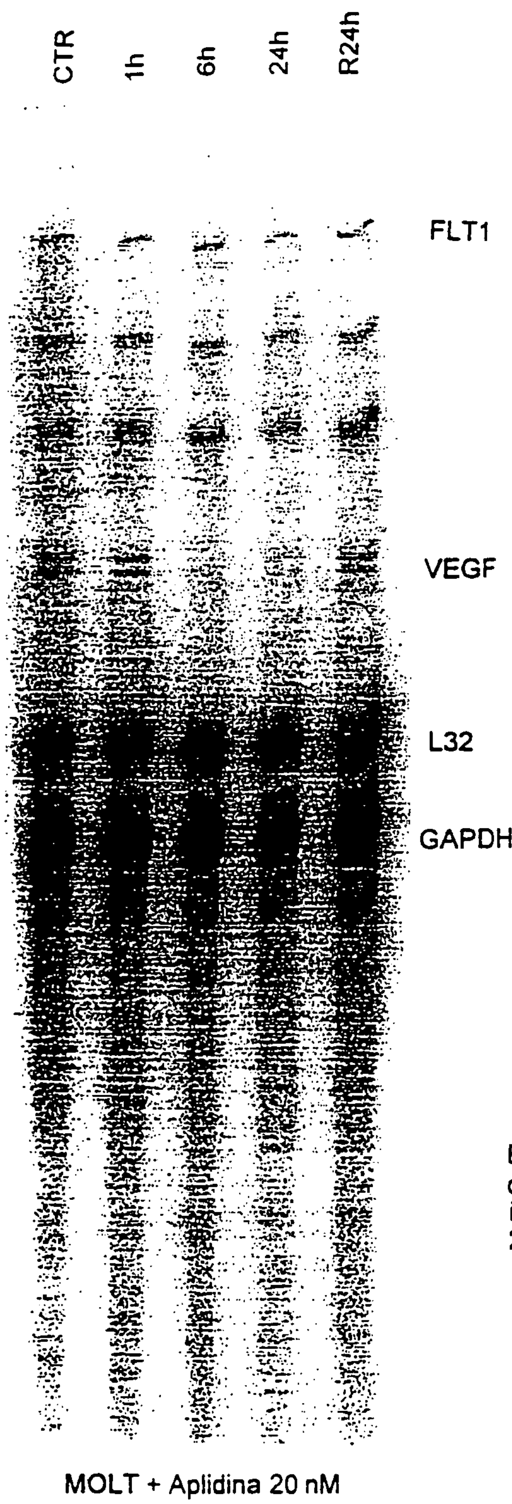


Fig.5



By using RNase protection, a strong decrease in the levels of VEGF mRNA in MOLT-4 cells was observable after 20nM aplidine

Fig.6

Aplidine does not decrease the activity of VEGF promoter transfected in MOLT-4 cells.

Cells were transfected with the VEGF promoter (spanning the first 1000 bases upstream the starting site linked to luciferase reporter gene and with a control plasmid containing the renilla reporter gene. Cells were then treated with different concentrations of aplidine and luciferase activity was measured after 24 hours and compared to renilla activity.

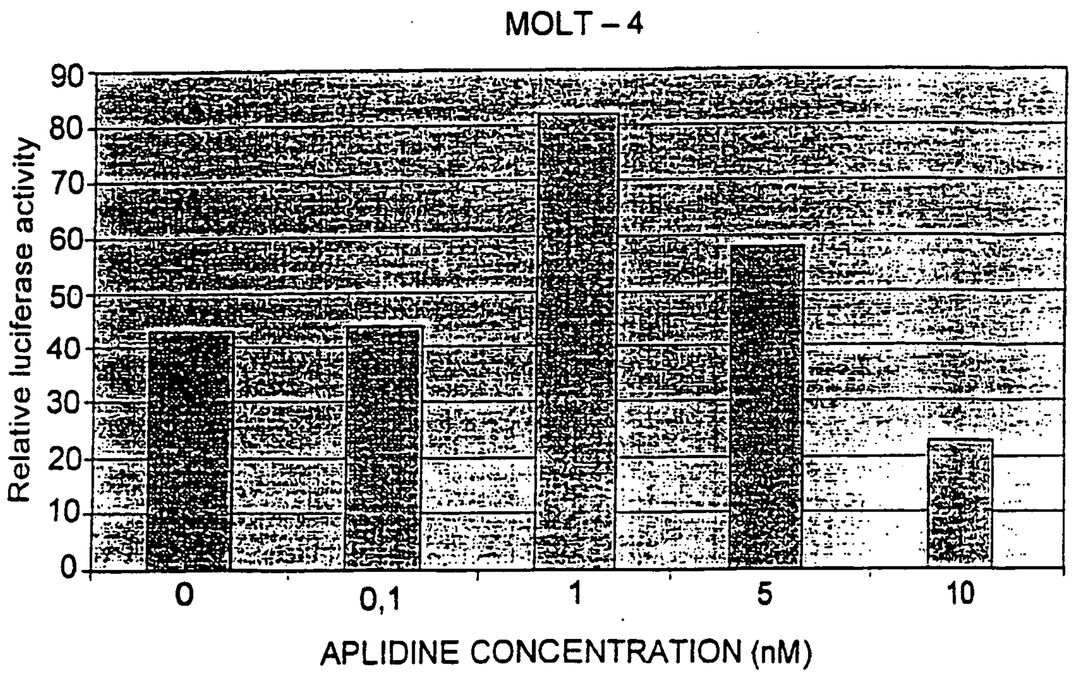
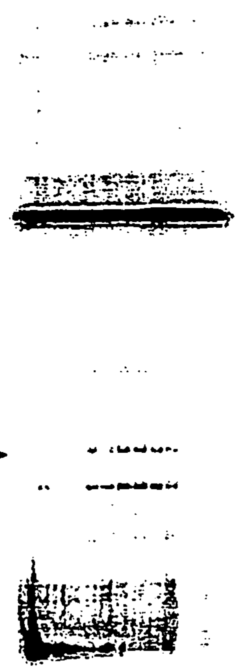


Fig.7

Aplidine does not block the binding of HIF-1 transcription factor to their concencus DNA sequences present in the VEGF promoter. Nuclear extracts were incubated with with diferent aplidine concentrations and labeled oligonucleotides for 60 min. Using gel retardation assay the binding of HIF-1 or AP-1 has been monitored.

HIF-1 →

AP-1 →

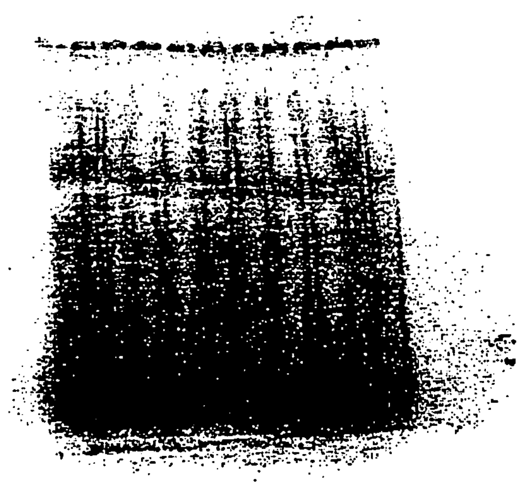


Lane 1 Control
Lane 2 Control + specific competitor
Lane 3 Control + unspecific competi tor
Lane 4 1 nM Aplidine
Lane 5 10 nM Aplidine
Lane 6 100 nM Aplidine
Lane 7 1 µM Aplidine
Lane 8 10 µM Aplidine
Lane 9 100 µM Aplidine

Fig.8

Aplidine does not block the binding of HIF-1 transcription factor to their concencus DNA sequences present in the VEGF promoter. Nuclear extracts obtained from cells treated with different aplidine concentrations were incubated with labeled oligonucleotides for 60 min. Using gel retardation assay the binding of HIF-1 has been monitored.

HIF-1 →



Lane 1 Control
Lane 2 Aplidine 1 nM 1 hour
Lane 3 Aplidine 1 nM 6 hours
Lane 4 Aplidine 1 nM 24 hours
Lane 5 Aplidine 5 nM 1 hour
Lane 6 Aplidine 5 nM 6 hours
Lane 7 Aplidine 5 nM 24 hours
Lane 8 Aplidine 10 nM 1 hour
Lane 9 Aplidine 10 nM 6 hours
Lane 10 Aplidine 10 nM 24 hours
Lane 11 Control

Fig.9

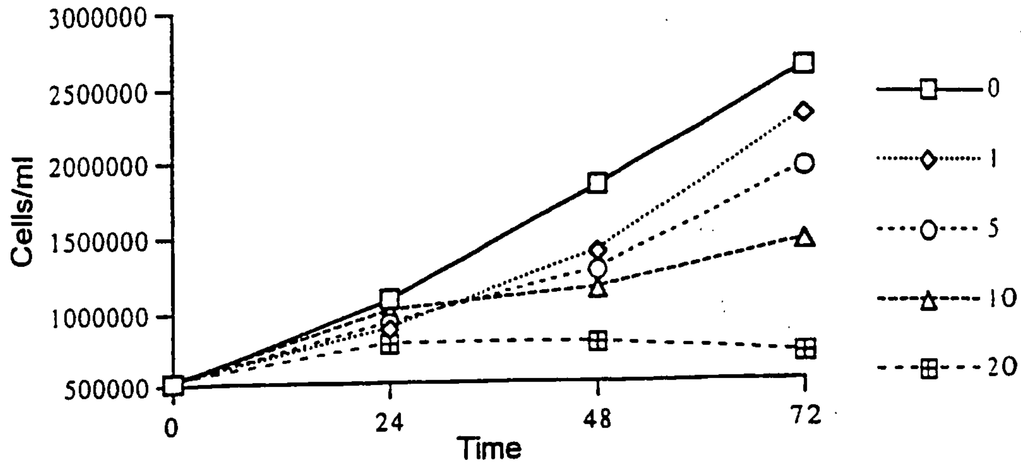


Fig.10a

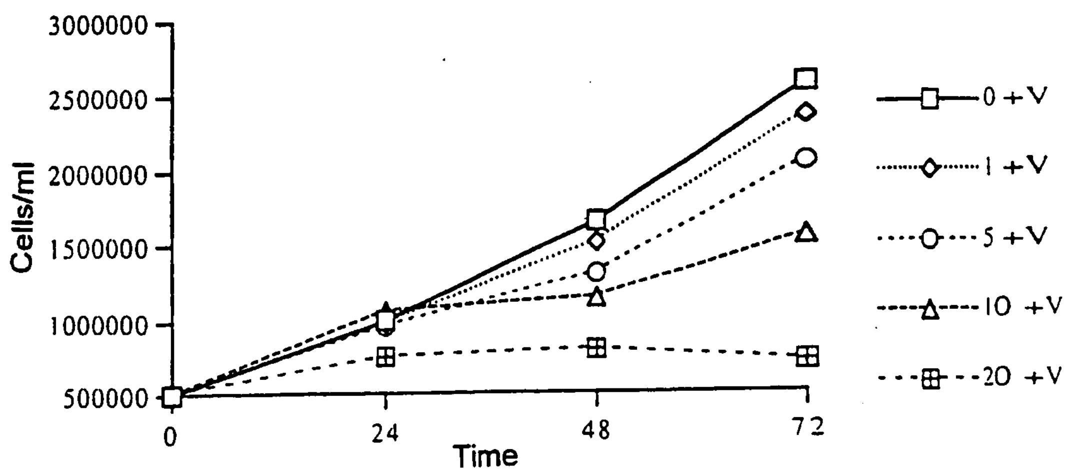


Fig.10b

Aplidine is also able to reduce the secretion of VEGF from the human ovarian cancer line IGROV – 1

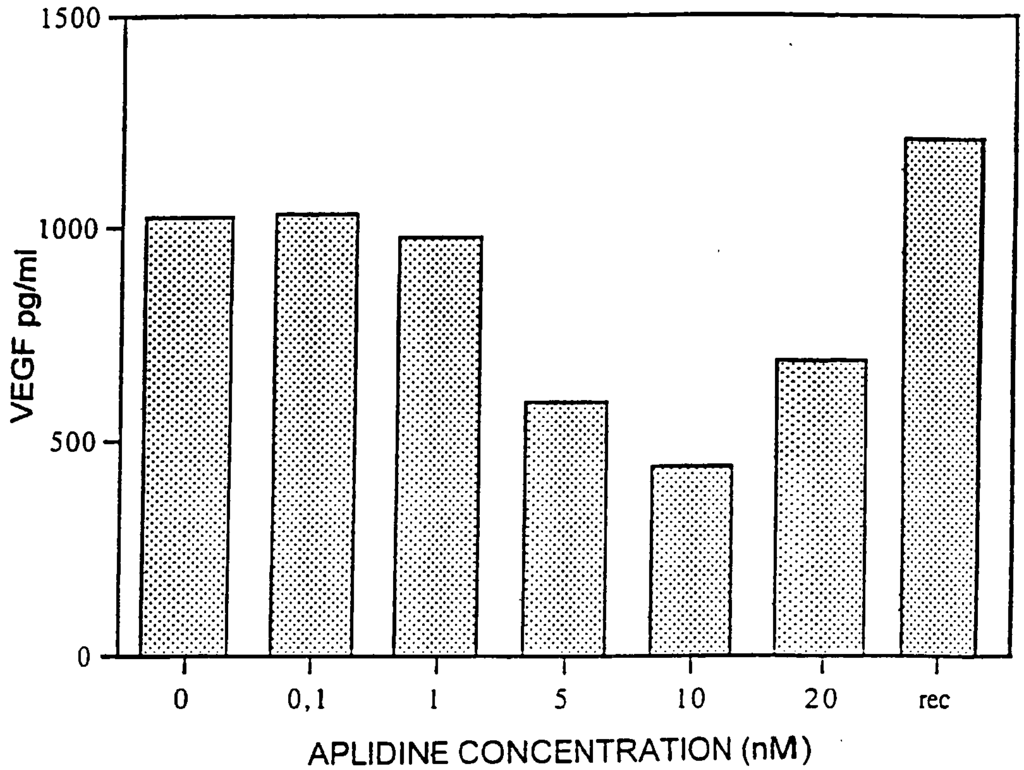


Fig.11

Aplidine reduces the mRNA levels of VEGF
also in the human ovarian cancar line IGROV - 1

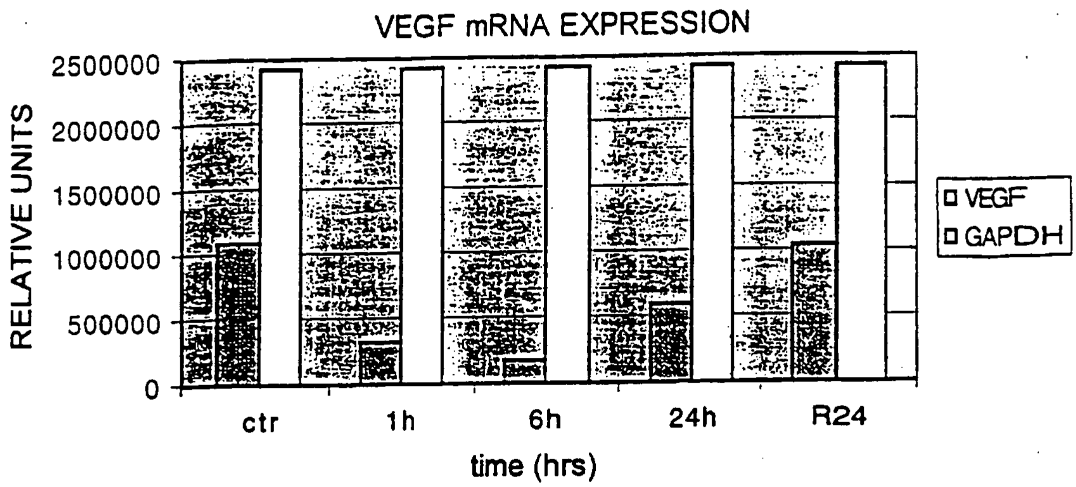


Fig.12

Aplidine does not affect the promoter activity of VEGF
measured using the luciferase/renilla reporter gene system

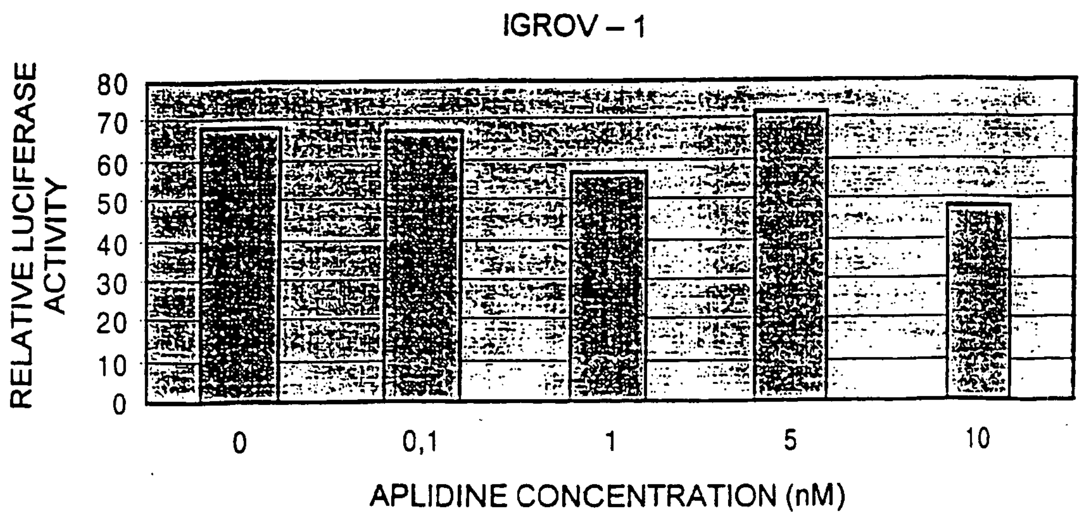
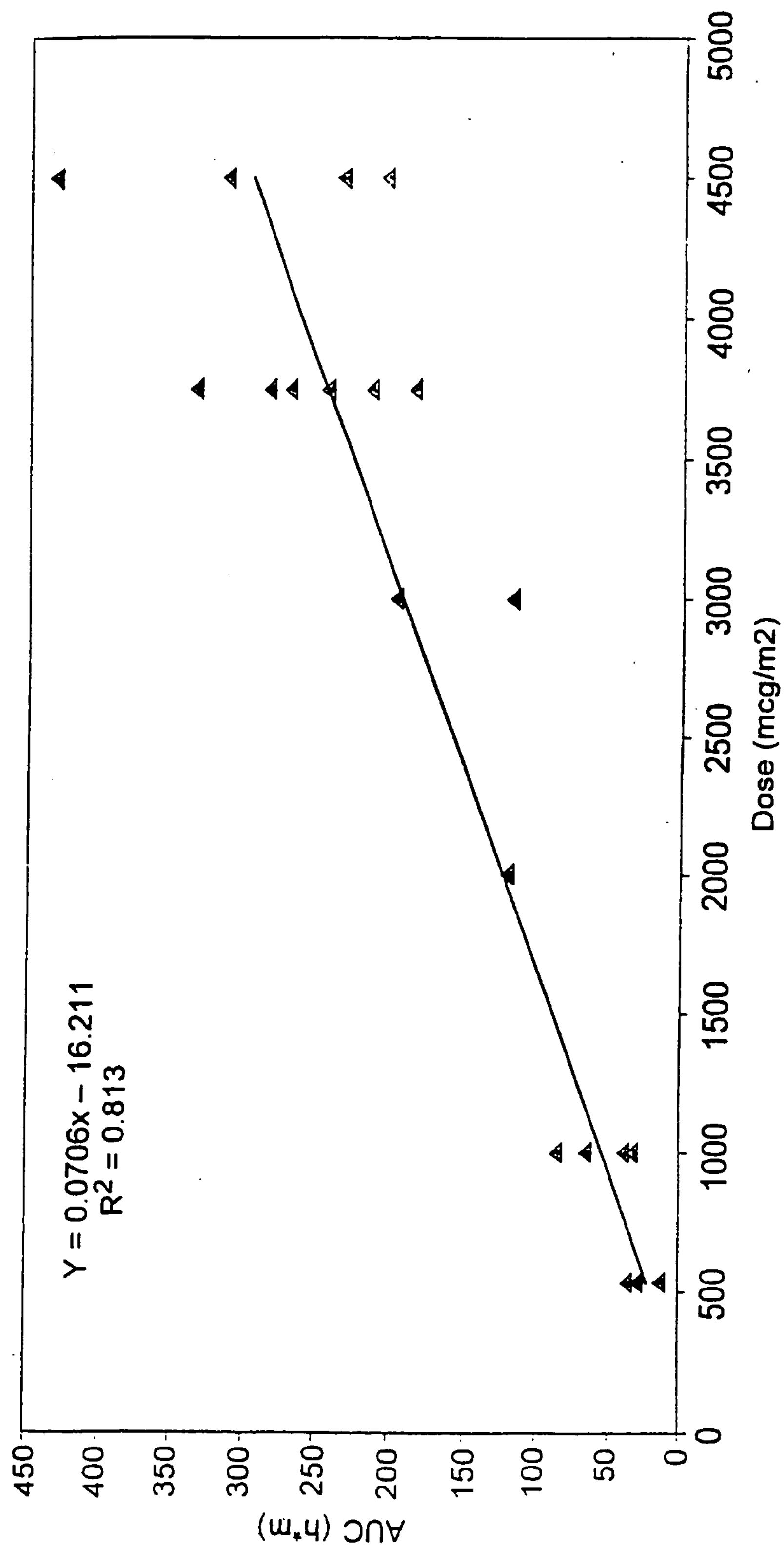


Fig 13



STUDY APL-A-001-98-DOSE AUC RELATIONSHIP

Fig.14

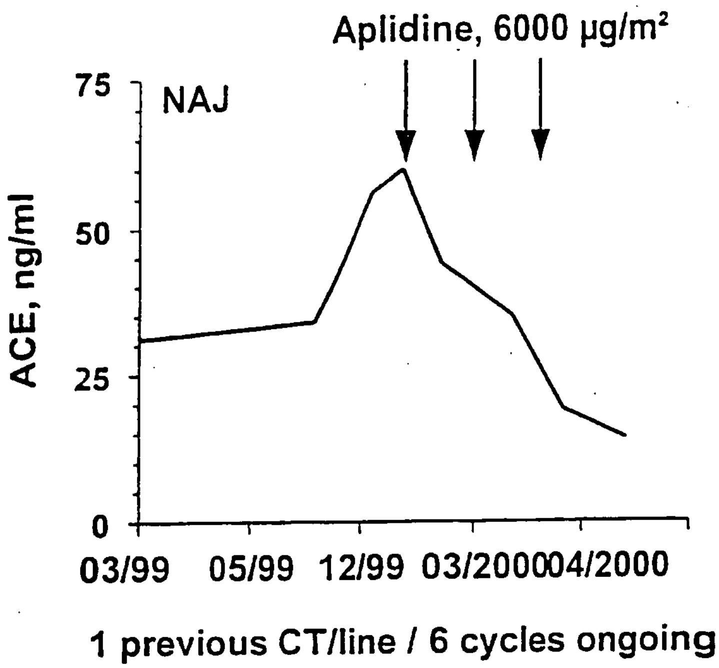


Fig 15a

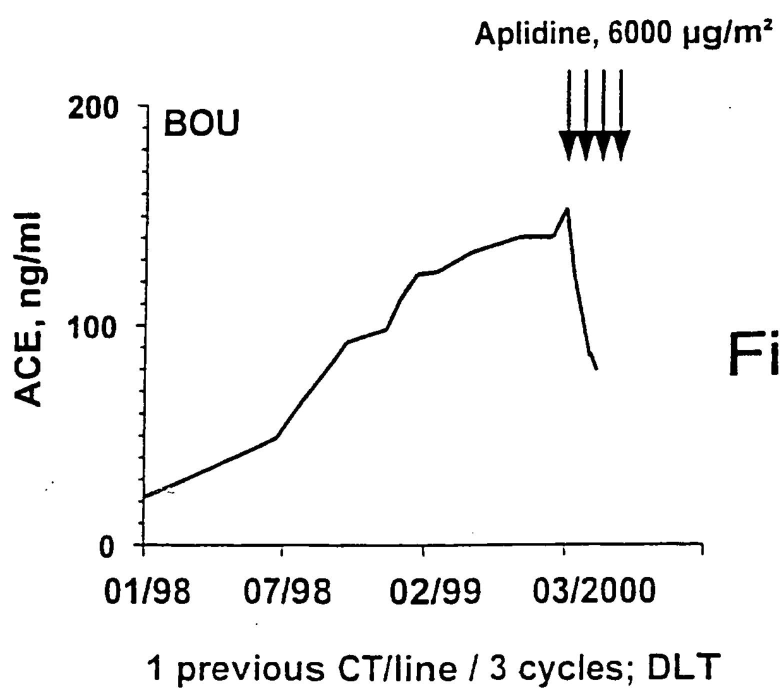


Fig.15b

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